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13. ABSTRACT (Maximum 200 Words)

The goal of this research project is to identify molecular changes that are associated with the progression of a peripheral nerve sheath tumor (PNST) from benign to malignancy. Archival and prospectively acquired benign PNSTs and malignant PNSTs are collected, and molecular changes at the *NF1* locus and throughout the genome are assessed. In preliminary findings, archived dermal neurofibromas from 5 patients and 2 benign PNSTs from 2 patients have been analyzed. It was found that the genome from the dermal neurofibromas is stable, with exceptions of allelic imbalance at 2/39 markers. There was evidence of allelic imbalance and loss of heterozygosity (LOH) in 4/39 markers from 2 sites in 1 benign PNST. The other benign PNST showed changes in 22/39 markers including LOH at the *NF1* locus. In contrast, no somatic inactivating deletions of the *NF1* gene were identified in the 38 dermal neurofibromas. Preliminary microarray expression data has been obtained prior to the beginning of this study for changes in gene expression between malignant and benign PNSTs. As part of this study, two sets of data were compared, and a set of candidate genes with altered expression have been identified but not validated. Finally, techniques for genotyping from microdissected tumor sections have been developed for use on MPNSTs when collected prospectively.

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INTRODUCTION

Peripheral nerve sheath tumors (PNSTs) are soft tissue tumors that are associated with neurofibromatosis type 1 (NF1). These tumors can undergo malignant transformation, however the mechanisms that are involved in this process are not known. The goal of this research project is to identify the molecular changes that are associated with the progression of a PNST from a benign to a malignant state. To carry out this project, archival and prospectively acquired PNSTs from NF1 individuals are examined for consistent genetic changes. The changes examined are identification of somatic mutations of the normal *NF1* allele, allelic imbalance analysis of genome-wide markers, microarray analysis of gene expression between peripheral nerve sheath, plexiform neurofibromas and malignant PNSTs, and mutation analysis of the *RAS* and *TP53* genes.

BODY

ARCHIVAL SPECIMENS
Identify genetic changes associated with malignant transformation in archival specimens
<u>Task 1:</u> Months 1-15   Obtain tumor specimens and perform pathology studies

The University of Utah and Department of the Army provided human subjects approval for this study in August 2000.   Tumor specimens are currently being identified and collected. Pathology sections are being prepared for special immunohistochemistry.

<u>Task 2:</u> Months 1-24   Select and begin microdissection across transition zones

Microdissection results from archival specimens are pending upon the evaluation by genomic allelic imbalance analysis and microarray analysis of whole tissue.

<u>Task 3:</u> Months 4-30   Genotyping and allelic imbalance analysis of microdissected DNA

Microdissection results on malignant archival specimens are pending, so no allelic imbalance analysis has been started.

<u>Task 4:</u> Months 12-30 Statistical analysis of allelic imbalance analysis in candidate regions

The detailed statistical analysis has not yet begun. Tumors are now being collected.

<u>Task 5:</u> Months 18-34 Expand allelic imbalance analysis in candidate regions

Candidate regions not yet identified.

<u>Task 6:</u> Months 6-18   Screen known candidate genes for mutations

The assays for the candidate genes are currently being optimized. It is planned that a PCR product will be generated from genomic DNA for exons 4-8 of *TP53*, which will then be sequenced.   At this stage the PCR reaction is being optimized (Figure 1 of appendices).

<u>Task 7:</u> Months 24-36 Screen candidate genes identified in this proposal

Candidate genes have not yet been identified experimentally, so screening has not yet begun.

Establish the degree of tumor heterogeneity in archival PNSTs

Task 8: Months 1-15   Obtain specimens and select foci for microdissection

Two benign PNSTs (36670 and 38628) have been acquired for analysis by microdissection (Figure 2, appendice). Microdissections have been performed on these 2 benign PNSTs, as well as 5 dermal neurofibromas from one patient. The method for microdissection is as follows. LCM enables the transfer of a few cells to an ethylene vinyl acetate film, which is activated by a laser diode pulse. LCM was performed on uncovered 5μm sections of tissue that were stained with haematoxylin and eosin (H&E). The cells of interest were then microdissected onto the film using 1000 shots. The cells were then digested using proteinase K at 55°C overnight. Final concentration of components were: 50mM Tris-HCl, pH8.5; 1mM EDTA; 0.5%(v/v) Tween-20; and 50μg/ml proteinase K. Samples were then boiled at 100°C for 10min in order to inactivate the proteinase K. Samples were then analyzed by polymerase chain reaction (PCR) by standard techniques. PCR products were quantified by densitometry and analyzed for allelic imbalance or LOH. The data from dermal neurofibromas have been included with a manuscript. Please see appended manuscript.

Task 9: Months 4-12   Perform genotyping and allelic imbalance analysis

Genotyping of DNA for positions 702, 2034 and 10647 in the *NF1* gene for 36670 and 38628 identified them as being either homozygous for the *NF1* gene, or having a deletion of the *NF1* gene (Figure 3, appendice). FISH analysis has not yet been performed to determine whether the patients do have a deletion of the *NF1* gene or not. In addition, 3/5 patients with only dermal neurofibromas were also determined to be homozygous. By fluorescence *in situ* hybridization (FISH) analysis, it was further determined that all 3 had a large deletion in the *NF1* gene. Refer to the appended manuscript. Gel-shift analysis of the *NF1* gene in the 2 benign PNSTs has not yet been performed. However, the 38 dermal neurofibromas from the 5 patients were analyzed and no somatic mutations that could have been insertions or deletions were detectable by size-shift at a 3-base resolution. Finally, for 38628 LOH has been observed in the marker Alu that is found within the *NF1* locus (Figure 4, appendice).

Allelic imbalance analysis was performed on the 2 benign PNSTs (Table 1, appendice). See Figure 5 of appendice for chromosomal location of polymorphic markers used in the allelic imbalance analysis. The results indicate for 36670, that 4/39 markers showed an allelic imbalance, with 1 being a LOH at UT5150 near the centromere of chromosome 11. None of the markers were lost in both areas of the same PNST from 36670, indicating heterogeneity within the tumor. The results from 38628 show marked changes across the genome, with 22/39 markers showing allelic imbalance and 10/22 being LOH. This could lead to the identification of important markers, but the data set is too small to be able to make definitive conclusions. In addition, 4 patients with a combined total of 19 dermal neurofibromas were also examined. Those results are included in the appended manuscript. The results show that the dermal neurofibromas are very stable for the majority of markers tested. There was one neurofibroma that showed an allelic

imbalance in UT40 found on chromosome 17q, and 3 neurofibromas had an allelic imbalance in UT913 found on chromosome 9q.

Task 10: Months 12-18 Compare foci to establish the degree of tumor heterogeneity

To determine the degree of tumor heterogeneity, different pieces from the same tumor will be analyzed or different areas from the same tumor section will be microdissected. The analysis of the benign PNSTs has begun with one of the tumors. The allelic imbalance analysis described in task 9, indicates that there is tumor heterogeneity. Sections from different areas of the same tumor were genotyped by using the polymorphic marker UT40 (Figure 6, appendice). The results also demonstrate tumor heterogeneity, as one section has both alleles, while the other section has LOH. There are caveats to the data because the genome wide allelic imbalance requires results from more PNSTs, and the microdissection requires rigorous testing with other markers within the NF1 locus. In addition, sections from 5 dermal neurofibromas were also microdissected to examine heterogeneity between these tumor types. Those results indicated only 1/5 dermal neurofibromas had an allelic imbalance in UT40 indicating again that dermal neurofibromas are relatively stable genetically (manuscript, appendice).

PROSPECTIVE SPECIMENS

Identify common genetic alterations in prospectively acquired benign and malignant PNSTs
Task 11: Months 1-24 Obtain tumor/blood pairs

Tumor/blood pairs are currently being collected.

Task 12: Months 1-24 Perform pathology analysis on PNSTs

Pathology analysis has yet to be performed on the PNSTs.

Task 13: Months 1-34 Perform *NF1* germline mutation analysis on PNSTs

No germline NF1 mutation analysis has been performed on prospectively acquired PNSTs. However, neurofibromin mRNA is being sequenced from the blood of prospectively acquired patients with NF1. To sequence the neurofibromin mRNA, neurofibromin cDNA is synthesized and amplified by polymerase chain reaction (PCR), and the PCR products are then sequenced to identify mutations. The primer sets have been designed so that they overlap and so the *NF1* mRNA is divided into 11 separate PCR products, each about 1kb in size (Figure 7, appendice). To date there is variation among the quality of the PCR products made for sequencing, more than likely due to the primers as well as the quality of the synthesized cDNA. That is being addressed by designing more primers to improve the consistency and yield of the PCR products prepared for sequencing.

Task 14: Months 1-30 Genotype and perform allelic imbalance analysis blood/tumor pairs

No genotyping or allelic imbalance analysis has been performed on prospectively acquired PNSTs. Nevertheless, informative genetic markers have been selected from each chromosomal arm, and they have been shown to be robust in providing allele imbalance estimates.

Task 15: Months 1-18 Perform differential display by microarray analysis of PNST RNA

Differential display has not been performed on prospectively acquired PNSTs, however 2 archival samples were analyzed (Figure 8, appendice) comparing benign PNSTs to malignant PNSTs. The data is only preliminary but multiple genes were identified. It appears that the assay will work, and will be able to identify potential genes involved in the malignant transformation of PNSTs. However, it is not the primary screen for mutations, and initially will be used in conjunction with results of the allelic imbalance analysis to identify consistent genetic changes.

Task 16: Months 12-30 Identify candidates by combining allelic imbalance/differential display

Once data is acquired candidates will be identified.

Task 17: Months 12-36 Compare allele imbalance differences of benign versus malignant PNSTs

Once more PNSTs are collected, this analysis will also be started.

Determine the level of *Ras*-activation in prospectively acquired PNSTs
Task 18: Months 3-30 Perform *Ras*-activation assays on frozen samples of PNSTs

The *Ras* activation assays will be performed as the PNSTs are collected. Preliminary studies using specimens from archived frozen tissue show estimates of *Ras* GTP/GDP ratios are obtainable. Dr. Gerry Boss provided this data via the consortium agreement. The specimens tested so far include 2 dermal neurofibromas, 17 plexiform neurofibromas and 6 different pieces of MPNST from 2 different individuals. See Figure 9 in the appendice.

Task 19: Months 12-36 Compare Ras GTP/GDP levels between benign and malignant PNSTs

The Ras GTP/GDP ratios obtained so far indicate that the dermal neurofibromas have the lowest GTP/GDP ratio (Figure 9, appendice). The GTP/GDP ratio from the plexiform neurofibromas and the malignant PNSTs are higher, however due to the small sampling of dermal neurofibromas there may not be a significant difference. There is also a lot of variability observed in GTP/GDP ratios from the plexiform neurofibromas. However,

there does not appear to be a significant difference between benign and malignant PNSTs. These results could be a result of the age of the specimens, because the specimens used were archival. More reliable results should be obtainable with the prospectively acquired specimens, that will be processed identically after arrival.

Determine somatic mutation status of candidate genes in PNST progression
Task 20: Months 1-30 Perform mutation analysis on known candidate genes

The mutation analysis will be performed on *TP53* and N*Ras*, H*Ras* and *KRas* as the PNSTs are acquired.

Task 21: Months 18-36 Perform mutation analysis on candidate genes identified in this study

Once identified the candidate genes will be analyzed.

Task 22: Months 12-36 Compare mutation status of candidate genes in benign versus MPNSTs

Once acquired, the mutation status of candidate genes in benign versus MPNSTs will be compared.

Develop for future NF1 investigators
Task 23: Months 1-18 Trainee develops, revises and streamlines protocols

Dr. Michael Liew started work on this project, June 1st, 2000. He is currently developing, revising and streamlining all protocols. He also attended the NNFF International symposium at Aspen, CO from June 4th-7th, 2000.

Task 24: Months 12-36 Trainee analyzes likelihood of non-NF1 loci role in PNSTs

Dr. Michael Liew will analyze the likelihood of non-NF1 loci in the role of PNSTs, once all data has been collected.

KEY RESEARCH ACCOMPLISHMENTS

- Inactivating somatic deletions of the *NF1* locus in dermal neurofibromas were not identified.
- Allelic imbalance in UT913 (chromosome 9q) identified in whole tissue extracts from 3 out of 19 dermal neurofibromas.
- Allelic imbalance in UT40 (chromosome 17q) identified in whole tissue extracts from 1 out of 19 dermal neurofibromas.
- Microdissected areas from 1 out of 5 dermal neurofibromas showed imbalance in UT40.
- A set of genome wide genetic markers has been shown to be robust for genotyping.
- Collection of tumor has been shown to be adequate for Ras biochemical assays.
- Mutation analysis of NF1 cDNA by sequencing has been developed as a pilot.
- PCR amplification of DNA from microdissected samples is shown to be effective for a subset of informative markers.
- Primer sets for *TP53* and *Ras* genes are developed for screening.
- Protocols for tissue handling have been developed.
- Approval for acquisition of prospectively acquired tumor has been obtained from 2 IRBs for overseeing human subjects research.

REPORTABLE OUTCOMES
Manuscript

A THOROUGH SEARCH OF THE *NF1* GENE FOR BOTH SOMATIC AND GERMLINE MUTATIONS

Bryan Allen, Michael Liew, Katsumi Tanito, Yan Zhang and David Viskochil

Poster presentation

Genome-wide Screen with Tetra-nucleotide Repeats Demonstrates that Allelic Imbalance in Dermal Neurofibromas is Rare. David Viskochil[1], Yan Zhang[1], Linda Ballard[1], Shunichi Sawada[2], Michihito Niimura[2]. [1]University of Utah, Salt Lake City, Utah; [2]Jikei University, Tokyo, Japan.

We have developed a genome-wide tetra-nucleotide genotyping screen to evaluate allelic imbalance in DNA derived from fresh-frozen tumor tissue. In an initial screen, we examined 19 dermal neurofibromas from 4 individuals with NF1. Tumors were surgically excised and divided into two halves, ½ was immediately frozen in liquid nitrogen and the other ½ was fixed for histopathological review. DNA was extracted from the frozen tumor and subjected to automated genotype analysis. Genotyper™ software was used to measure the area under the peaks and the ratio of the areas for two alleles was compared between blood and tumor DNA to estimate allelic imbalance in tumor-derived DNA template. Given the small sample size, a ratio of peak areas for informative alleles that was either less than or greater than 0.3 relative area ratio in tumor versus blood DNA samples was scored as allelic imbalance. This allele imbalance could reflect either loss of heterozygosity or amplification. Histological analysis of each tumor revealed significant homogeneity, which suggests that cells that could harbor allelic imbalance would not have been diluted by allelic balance expected in normal cells derived from the tumor. The tetranucleotide marker map sites were selected from distal chromosome arms to detect chromosomal instability in dermal neurofibromas.

We genotyped 40 markers from 22 chromosomes. Chromosomes 2q and 20p were not represented in the screen. The markers were informative in 134 assays. There were 22 instances of allelic imbalance in 720 genotypes from the 19 neurofibromas. Sites of imbalance included; 6p (1), 6q(1), 7(2), 8p(1), 9q(6), 11p(6), 13(3), and 17q(2). These data suggest that allelic imbalance is not common in dermal neurofibromas. Sites that may be commonly altered include chromosomes 7, 9q, 11p, 13, and, not unexpectedly, 17q. Use of these markers with adjacent sets of markers in an extended set of tumors would better define candidate genes that may contribute to neurofibroma formation and growth.
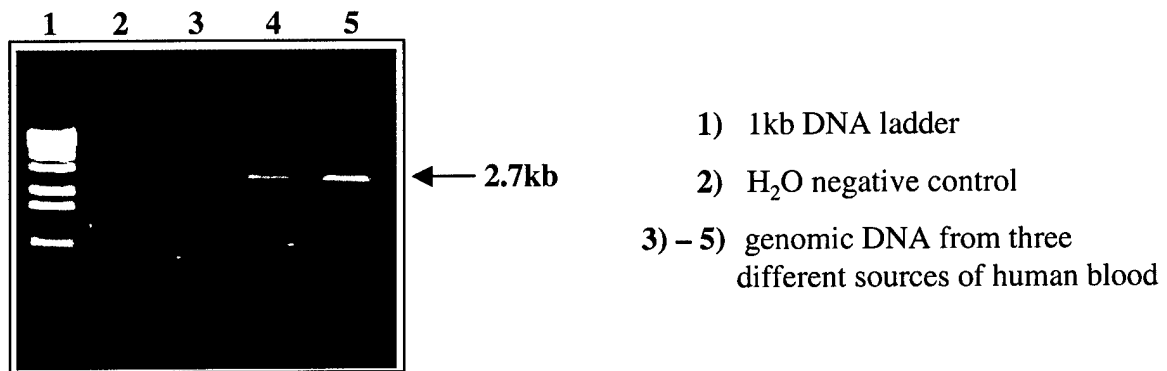
CONCLUSIONS

The main accomplishment of the project so far has been the development of the necessary protocols for examining the prospectively acquired benign and malignant PNSTs. The sequencing of the *NF1* mRNA initiated in this project is important because it will eventually provide a high throughput means of identifying mutations in the *NF1* gene. The development phase has been slow, due to the difficulty in designing primers that give an adequate yield of specific product from cDNA synthesized from blood RNA. Future work includes designing more primers that result in higher yields of PCR product for sequencing.

The data compiled so far on the benign PNSTs indicate that heterogeneity within a tumor can be detected. The data also indicates that there are differences between benign PNSTs, because one of them also had multiple sites of LOH. This probably means that there are other possible genetic changes outside of *NF1* that may have lead to their development, and that perhaps some of the markers could indicate a progression in the tumor. However, it must be stressed that at this point the data is not sufficient by itself to make valid conclusions. But with the protocols now in place, the project is poised to identify the genetic changes that occur in the change of a benign PNST into a malignant PNST.

APPENDICES

Figure 1. PCR amplification of *TP53* from genomic DNA

← 2.7kb

1) 1kb DNA ladder

2) H$_2$O negative control

3) – 5) genomic DNA from three
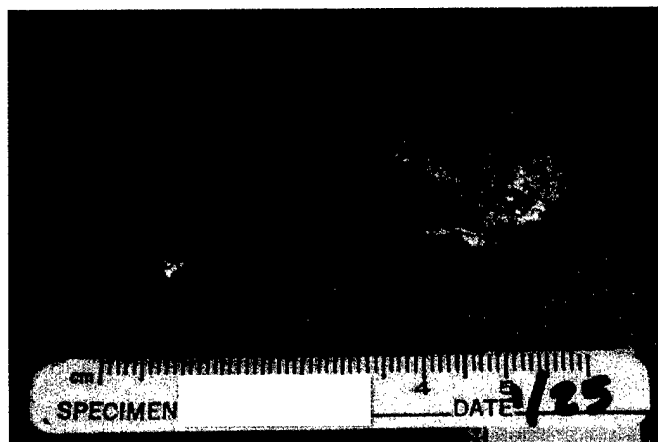different sources of human blood

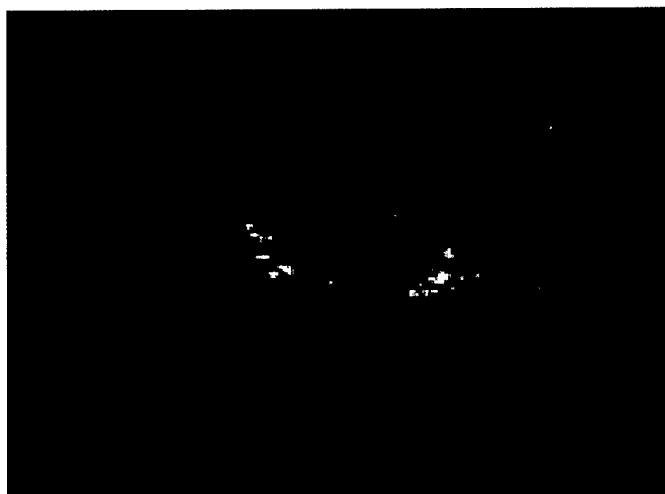Primer sequence used for PCR amplification of *TP53*:

6a: agcgaaaattcatgggactg ⟶

8r: taactgcacccttggtctcct ⟵

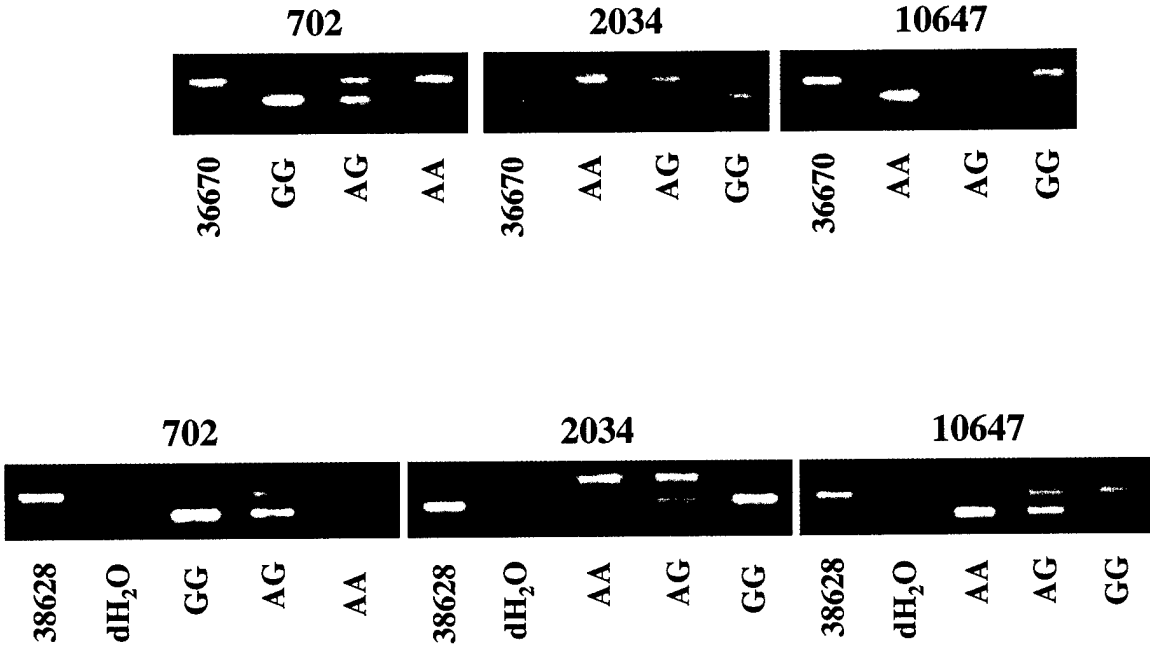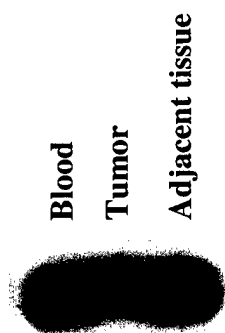**Figure 2. Benign PNSTs from patients 36670 and 38628**

36670

38628

Figure 3. Genotyping of positions 702, 2034 and 10647 in the NF1 gene in patients 36670 and 38628

**Figure 4.** LOH at the *NF1* locus in a benign PNST from patient 38628.

Table 1. Genotypes of 2 benign PNSTs from 2 patients.

The markers used are indicated along the side of the table, while patient samples are across the top of the table. Symbols used are as follows. = allelic balance; - not determined; F failed sample; I allelic imbalance; LOH loss of heterozygosity; N not informative.

Patient	36670(assay1)			36670(assay2)			38628		
Marker	Blood	Tumor 1	Tumor 2	Blood	Tumor 1	Tumor 2	Blood	Adjacent tissue	Tumor
UT5144-1p		=	=		-	-		=	LOH
UT5170-1q		=	LOH		=	I		N	N
UT595-2p		=	F		-	-		=	=
UT1360-3p		=	=		-	-		=	=
UT6129-3q		N	N		-	-		N	N
UT878-4p		=	=		-	-		=	I
UT615-5p		N	N		-	-		N	N
UT5013-5q		N	N		-	-		=	LOH
UT886-5q		=	=		-	-		N	N
UT2018-6p		N	N		-	-		=	I
UT897-6q		=	=		-	-		N	N
UT5189-7p		F	F		-	-		=	=
UT5412-8p		N	N		-	-		=	I
UT909-8q		=	=		F	=		=	I
UT873-9p		=	=		-	-		=	LOH
UT913-9q		F	F		-	-		F	F
UT7422-9q		=	=		-	-		N	N
UT1699-10p		=	=		-	-		N	N
UT5419-10q		=	=		-	-		N	I
UT8115-11p		N	N		-	-		I	LOH
UT5150-11		LOH	=		LOH	=		=	LOH
UT2095-11q		F	=		-	=		=	LOH
UT5029-12p		N	N		-	-		=	I
UT6136-12		-	-		-	-		=	=
UT931-12q		=	=		-	-		N	N
UT2413-13		N	N		-	-		=	LOH
UT1392-14		=	=		-	-		I	=
UT1232-15		=	=		-	-		=	I
UT581-16p		=	=		-	-		=	=
UT703-16q		=	=		-	-		=	I
UT269-17p		I	=		-	-		=	LOH
UT40-17q		=	=		-	-		=	I
UT7162-18p		N	N		-	-		F	F
UT576-18q		N	N		-	-		=	I
UT5187-19p		=	=		-	-		=	=
UT1342-19q		=	F		F	I		=	LOH
UT236-20		N	N		-	-		=	I
UT1355-20q		=	=		-	-		=	I
UT1091-22q		N	N		-	-		=	LOH

Figure 5. Distribution of markers on the 22 chromosomes used in allelic imbalance analysis of the human genome
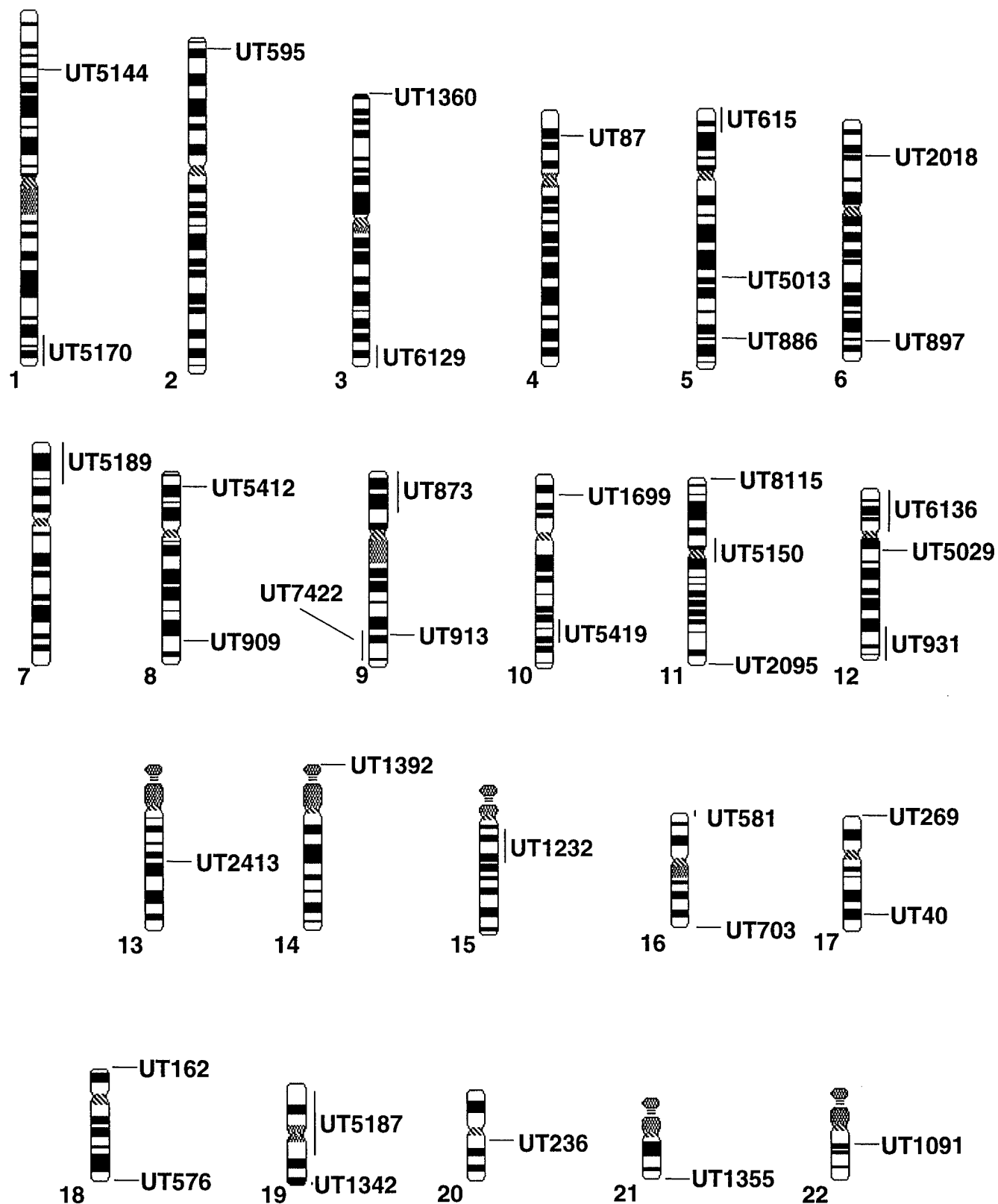
Figure 6. Analysis of UT40 from microdissected samples of benign PNST from patient 36670

Figure 7. Primers used to make 11 PCR products for sequencing of the NF1 cDNA

Black arrows indicate the start and end of the coding region of the NF1 cDNA. Same colored arrows are the pairs of primers and their relative position along the NF1 cDNA used to generate the 11 PCR products for sequencing. Product 1 spans exons 1-6, product 2 spans exons 4-10, product 3 spans exons 10-16, product 4 spans exons 15-20, product 5 spans exons 19b-24, product 6 spans exons 23-2-28, product 7 spans exons 27b-31, product 8 spans exons 30-35, product 9 spans exons 34-42, product 10 spans exons 40-48 and product 11 spans exons 44-49.
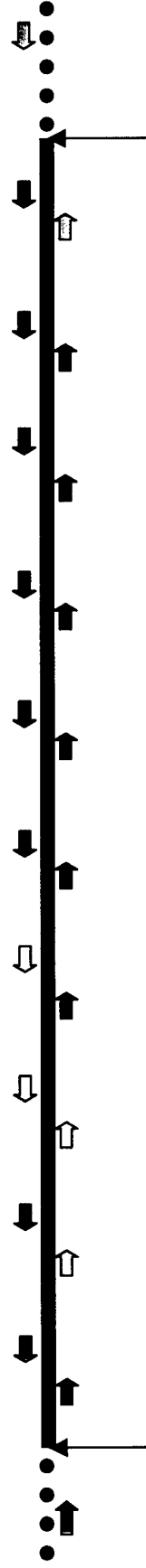
Figure 8. Differential display of expression of genes in a benign versus malignant PNST

Arrows indicate genes that have changed expression.
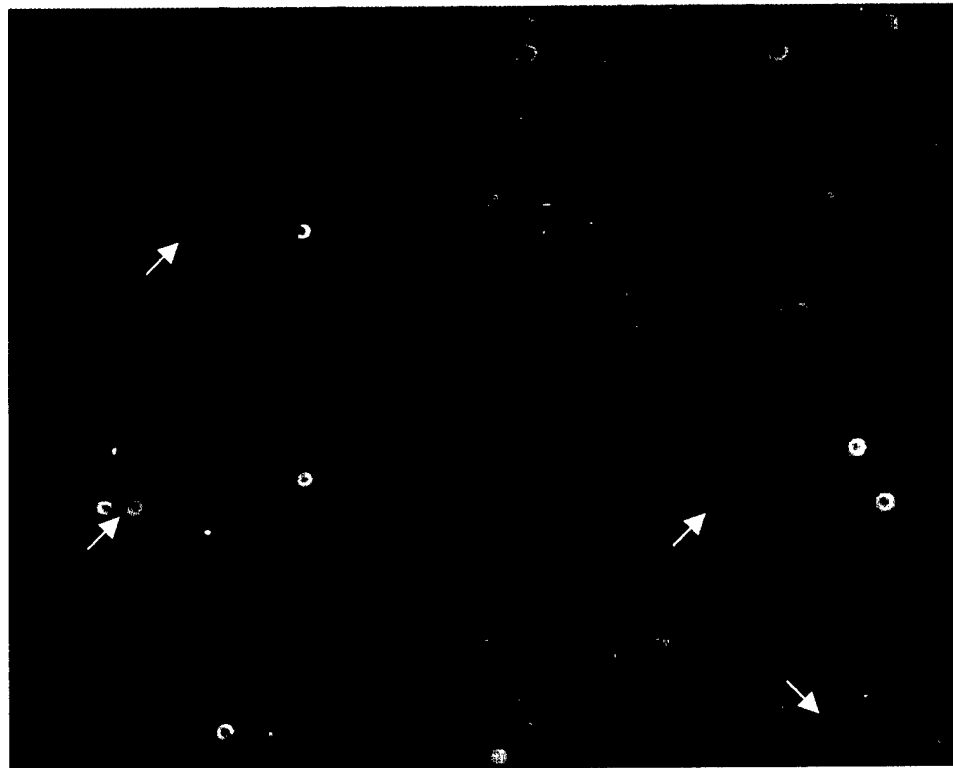
Figure 9. GTP/GDP ratios comparing dermal neurofibromas, plexiform neurofibromas and malignant PNSTs.

Data provided by Dr. Gerry Boss via the consortium agreement.
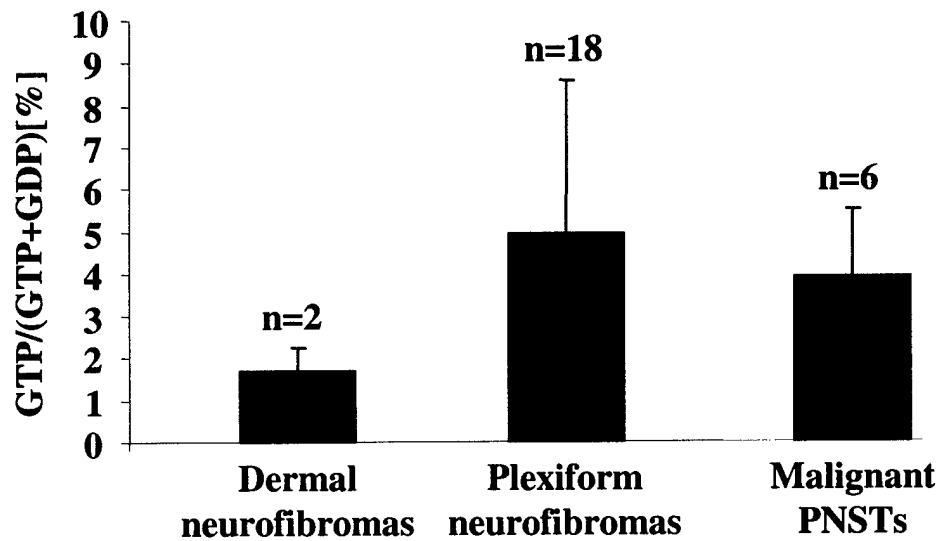
A THOROUGH SEARCH OF THE *NF1* GENE FOR BOTH SOMATIC AND GERMLINE MUTATIONS

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ABSTRACT

Neurofibromatosis Type 1 (NF1) is a common autosomal dominant genetic disorder that is caused by a mutation in the *NF1* gene. A feature of the disorder is the formation of benign tumors called neurofibromas. The precise genetic changes that lead to the formation of neurofibromas has not yet been identified. However, there are contradicting reports that the development of neurofibromas requires a constitutive mutation in the germline DNA plus a somatic mutation that occurred at a different time. This study set out to fate map dermal neurofibromas found on different parts of the body, but found no loss of heterozygosity (LOH) in 2 constitutional deletion patients with a combined total of 24 dermal neurofibromas. In addition, in 3 non-deletion patients no inactivating somatic mutations could be identified. In conclusion, in the patients studied, this study found no evidence of a second mutation that could have lead to the development of the dermal neurofibromas.

INTRODUCTION

Neurofibromatosis Type 1 (NF1) is an autosomal dominant genetic disorder that is caused by mutations in the *NF1* gene. The *NF1* gene was identified and sequenced in the early 1990's; it maps to location 17q11.2 and spans 60 exons. The gene is fairly large spanning approximately 350 kilobases and encodes a 2818 amino acid protein, neurofibromin. NF1 is characterized by the hallmark manifestations of café-au-lait spots and neurofibromas. The most common types of neurofibroma observed clinically are dermal neurofibromas which are benign focal lesions of schwann-like cells and fibroblasts associated with the sensory nerve tracts. Neurofibromas which involve large segments of nerve sheaths and nerve roots are called plexiform neurofibromas. Plexiform neurofibromas are usually benign, but a small percentage give rise to malignant peripheral nerve sheath tumors (MPNSTs)(Riccardi, 1992).

One proposed mechanism for tumor development is the "two hit" hypothesis (Knudson, 1971). In this scenario, the remaining normal allele gets inactivated in individuals that are heterozygous for a genetic predisposition to *NF1*, leading to tumor development. Previous studies have found that the somatic second hit is often a deletion of the chromosome region that contains the suppressor gene. Therefore, by screening the patient's DNA with polymorphic markers, and analyzing the exons of the *NF1* gene for size shifts, the somatic mutations generating a loss of heterozygosisty (LOH) may be detectable.

There is accumulating evidence that NF1 is a tumor suppressor gene. This has been demonstrated by the identification of somatic inactivating mutations of the *NF1*

gene in tumors from individuals with neurofibromatosis type 1. "Second hits" in NF1 have been observed as a loss of heterozygosity in juvenile myeloid leukemia, phaeochromocytomas and MPNSTs (Shannon et al, 1994; Xu et al, 1992; Legius et al, 1993; Lothe et al, 1995; Bollag et al, 1996). Double inactivation has been more difficult to demonstrate in dermal neurofibromas. Earlier studies did not identify a loss of heterozygosity (LOH) in dermal neurofibromas (Menon et al, 1990; Glover et al, 1991; Skuse et al, 1991; Lothe et al, 1995) but only in malignant NF1 tumors (Skuse et al, 1989; Legius et al, 1993).

More recently however, LOH has been demonstrated in neurofibromas and there have been reports of "two hits" to the NF1 gene (Colman et al, 1995; Sawada et al, 1996; Daschner et al, 1997; Serra et al, 1997; Alison et al, 2000; Eisenbarth et al, 2000). In addition the study by Sawada et al, (1996) provides a means for fate mapping clusters of neurofibromas in deletion patients. We were unable to identify LOH in 24 dermal neurofibromas from 2 constitutional deletion patients. Therefore, we expanded our screening protocols for somatic mutations and included 3 additional non-deletion patients. We were unable to detect inactivating somatic mutations which is more consistent with reports by Daschner et al, (1997) and John et al, (2000) who identified second hits in 2.6% and 12% respectively, of dermal neurofibromas screened. This report describes the techniques we used to screen dermal neurofibromas for somatic mutations. In addition, we screened the genome for allelic imbalance at other loci in a subset of dermal neurofibromas to demonstrate a lack of genomic instability in these benign tumors.

METHODS

Patient Identification

NF1 patients participating in this study met the criteria established at the consensus conference at the National Institutes of Health (Strump et al., 1987). Tissue samples used in this study were obtained with permission from the University of Utah Institutional Review Board. Five NF1 patients were used in this study, with a combined total of 38 dermal neurofibromas. The dermal neurofibromas obtained from patient B100, were from different areas of the body which corresponded to different spinal dermatomes (Figure 1).

Flourescence *in situ* hybridization (FISH)

FISH analysis was performed as described in Purandare, et al 1995.

DNA extraction

DNA extraction from whole blood was conducted following Gentra^{TM} specifications. 1 part of whole blood was mixed with 3 parts RBC lysis solutions. Invert to mix and incubate at room temperature for 10min. Invert again after 5min. Centrifuge at 2000g for 10min at room temperature. Discard supernatant, leaving 100-200µl of residual fluid on top of the pellet. Vortex vigorously and add 3ml of cell lysis solution. Add 1ml of

protein precipitation solution, vortex vigorously then centrifuge at 2000g for 10min at room temperature. Transfer supernatant containing the DNA into 3ml 100%(v/v) isopropanol. Mix by inverting gently 50 times and centrifuge at 2000g for 3min at room temperature. Wash the DNA pellet with 3ml 70%(v/v) ethanol and centrifuge at 2000g for 1min at room temperature, then air dry for 10-15min at room temperature. The DNA was then resuspended in 250µl sterile distilled water by incubating at room temperature overnight, then stored at –20°C.

Mutagenically separated PCR (MS-PCR)

MS-PCR studies were conducted as described by Purandare et al. (1996). This is a technique where polymorphisms can be detected in a mutant and normal allele, that can be amplified in the same tube by using different length allele specific primers. PCR reactions were carried out in 25µl reaction volumes with the following final concentrations/amounts of components. 50ng of total genomic DNA used as template, 1×PCR buffer (Gibco BRL), 20µM each dNTP, 0.25mM spermidine, 0.1%(w/v) Triton X-100, and 0.025 units of *Taq* DNA polymerase/µl. The final concentration of primers was as follows. For position 702, 0.5µM primer 52RC702, 0.125µM primer 53RC702 and 0.5µM primer I5RP. For position 2034, 0.5µM primer NPIS, 0.17µM primer 31RC2034 and 0.5µM primer 32RC2034. For position 10647, 0.4µM primer MS5'-1, 0.4µM primer MS3-T, and 0.05µM primer MS3-C. Thermal cycling conditions were 94°C for 5min; 94°C for 10s, 60°C for 10s, and 72°C for 10s, repeated for 5 cycles;

followed by 94°C for 10s, 56°C for 10s, and 72°C for 10s, repeated for 30 cycles. The PCR products were electrophoresed on a 4% (w/v, 3:1 Nusieve:Seakem LE) agarose gel.

PCR Mutation Analysis

PCR reactions looking for size shifts were conducted in one of two ways, either in a multiplex reaction where two or three primer sets were amplified in an individual PCR reaction or in a standard PCR reaction with only 1 primer set being amplified, both of which used radioactively labeled primers. The primers used were originally listed by Li et al., (1995), but are listed here again (Table 1). End labeling for both methods was conducted by incubating 5pmol of a 5' or 3' primer with $10\mu Ci\gamma^{32}P$-dATP (ICN biomedicals) with 3U of T4 Polynucleotide Kinase in 1×kinase buffer (GibcoBRL) at 37°C for 45mins. PCR amplification for both multiplex and individual amplifications was conducted in 25μl reaction volumes, each of which contained 50 to 250ng DNA, 1×PCR buffer (Gibco BRL), 50μM of each dNTP (USB), 2.5pmol of each cold primer, 0.3pmol end labeled primer, 400μM Spermidine, and 1U *Taq* DNA Polymerase (GibcoBRL). The amplification conditions were as follows: an initial denaturation at 94°C for 5min; forty cycles of 94°C for 30s, 54°C for 30s, and 72°C for 2min; and a final extension period of 5min at 72°C. PCR products were run on 6% polyacrylamide, 8.3M urea, TBE gels and visualized by autoradiography.

Genotype Analysis

Conducted as previously described by The Utah Marker Development Group (1995). DNA extracted from the dermal neurofibromas was compared to DNA from the blood of the same patient. In order for a marker to be imbalanced the normal/tumor ratio had to be either less than 75% or greater than 125%. For a marker to show a loss of heterozygosity, the ratio had to be less than 20% or greater than 500% (Paulson et al., 1999).

Laser Capture Microdissection (LCM)

LCM enables the transfer of a few cells to an ethylene vinyl acetate film, which is activated by a laser diode pulse. LCM was performed on uncovered 5μm sections of tissue that were stained with haematoxylin and eosin (H&E). The cells of interest were then microdissected onto the film using 1000 shots. The cells were then digested using proteinase K at 55°C overnight. Final concentration of components were: 50mM Tris-HCl, pH8.5; 1mM EDTA; 0.5%(v/v) Tween-20; and 50μg/ml proteinase K. Samples were then boiled at 100°C for 10min in order to inactivate the proteinase K.

For PCR reactions, 2μl was used for every 25μl of reaction volume. PCR reactions were carried out 5 times for the polymorphic markers UT40 and UT290. UT40 primers were end labeled by incubating 3pmol of a 5' or 3' primer with 8μCiγ^{32}P-dATP (ICN biomedicals) with 1U of T4 Polynucleotide Kinase in 1×kinase buffer (GibcoBRL). Reactions were incubated at 37°C for 45mins. PCR amplification for UT40 was conducted in 25μl reaction volumes, which contained 50ng DNA extracted from whole

tissue or 2μl of LCM sample, 1×PCR buffer (Gibco BRL), 35μM of each dNTP (USB), 1.5pmol of each cold primer, 0.3pmol end labeled primer, 400μM Spermidine, and 1U *Taq* DNA Polymerase (GibcoBRL). The amplification conditions were as follows: an initial denaturation at 94°C for 5min; forty cycles of 94°C for 30s, 54°C for 30s, and 72°C for 2min; and a final extension period of 5min at 72°C. PCR products were run on 6% (v/v) polyacrylamide, 8.3M urea, TBE gels and visualized by autoradiography. To test for LOH band densities were quantified by densitometry using the software program Imagequant.

RESULTS

MS-PCR

Results from the MS-PCR analysis revealed that patients B100, U100 and U125 were homozygous at all three positions tested (Figure 2). Meanwhile patients U129 and U135 were heterozygous. Patient U129 was AG at position 702 and patient U135 was AG at position 10647.

Fluorescence *in situ* hybridization (FISH)

FISH analysis was performed on the 3 patients that were identified as homozygous at the *NF1* gene. The analysis was performed on B100, U100 and U125. The results suggested that either part of one *NF1* allele, or one entire *NF1* allele had been deleted in all 3 patients.

PCR mutation analysis

All 38 dermal neurofibromas were examined for somatic size shift occurrences in each of the 60 exons of the *NF1* gene. However, no detectable size shift was apparent in any of the exons examined in any of the dermal neurofibromas (Figure 3).

LCM analysis

To determine if there was any possible LOH on chromosome 17 sections from dermal neurofibromas obtained from patient U135 were microdissected and the polymorphic marker UT40 was analysed for a loss of heterozygosity. The histological sections of the dermal neurofibromas were very uniform in staining (Figure 4). As an example, the areas microdissected from dermal neurofibroma D are shown. Densitometry of the PCR products revealed that there was an imbalance in UT40, this time in dermal neurofibroma D (Figure 5). However, there was another area of neurofibroma D that did not show an imbalance of UT40. Only one area of neurofibroma B was available for LCM analysis.

Genotype analysis

Genotype analysis was performed using 39 markers spread out across 22 chromosomes. Nineteen dermal neurofibromas from 4 of the 5 patients were included in the genotyping, because DNA from the blood of one patient was not available. A set of 5 intragenic and 34 extragenic polymorphic markers were used (Table 2). Of the markers tested, none demonstrated a clear loss of heterozygosity. However, 4 neurofibromas from 3 different patients demonstrated an allelic imbalance. Neurofibroma E from U135 had an allelic imbalance in UT40 found on chromosome 17q. Neurofibroma B from U129 and neurofibromas B and D from U100 had an allelic imbalance in UT913 found on chromosome 9q.

DISCUSSION

Neurofibromin is structurally related to members of the Ras-GTPase activating protein family. The NF1 GAP-related domain (NF1-GRD), found in exons 20-27a, is highly conserved. RAS-GAPs negatively regulate RAS signal transduction (Boguski and McCormick, 1993). It has been demonstrated that MPNST cell lines lacking neurofibromin have elevated levels of RAS-GTP, which leads to uncontrolled growth (DeClue et al, 1992; Basu et al, 1992; Guha et al, 1996). Therefore, in general, inactivation of *NF1* leads to an increase in cell proliferation because there is decreased neurofibromin and decreased negative regulation of RAS signaling. These observation support the hypothesis that *NF1* acts as a tumor suppresser gene (DeClue et al, 1991).

This study began with the aim to develop a fate map for tumor development from a patient known to have lost one entire *NF1* allele. A previous study using a similar approach identified a 4 base-deletion in an NF1 patient with a deleted *NF1* allele (Sawada et al. 1996). However, the analysis in this study did not identify any changes in the *NF1* gene that could have accompanyed the development of neurofibromas in different parts of the body. As a result, the study was expanded to include four additional NF1 patients. Two of the patients had a known germline mutation which was a deletion of one *NF1* allele. These patients also did not show any sign of somatic mutations that were deletions of the exons of the *NF1* gene.

There are widespread but varying reports of identification of a loss of heterozygosity in the *NF1* gene as the second hit. Some studies have not identifed LOH in dermal neurofibromas (Menon et al, 1990; Glover et al, 1991; Skuse et al, 1991; Lothe et al, 1995). However, Coleman *et al.* (1995) found that 8 out of the 22 neurofibromas

that they analyzed showed somatic deletions involving the *NF1* gene while Daschner *et al.* (1997) observed LOH in only 1 out of the 38 neurofibromas they analyzed. The most recent study by Rasmussen *et al.* (2000) demonstrated that 2/15 dermal neurofibromas had LOH, however did not classify it as the second hit.

A genotype analysis searching for allelic imbalance throughout the genome was conducted in the tumors from the same four patients. Allelelic imbalance was only observed in 2/39 markers tested from 4/19 dermal neurofibromas. Of those only 1 neurofibroma showed an imbalance in chromosome 17, right near the telomere. This suggests that the entire genome in dermal neurofibromas is relatively stable.

The difference between the results obtained from the LCM and the genotype analysis was probably due to the differences in the source of DNA. The genotype analysis required a large piece of neurofibroma while the LCM extracted DNA from a couple of thousand cells. Therefore, because any type of tumor demonstrates heterogeneity in cell clonality, LCM analysis would be able to detect more subtle genetic changes. This seems to be the case in our study of dermal neurofibromas. The DNA extracted from a piece of neurofibroma tissue showed no allelic imbalance, while DNA extracted from one of the LCM sections demonstrated allelic imbalance.

The results from this study suggest that a second hit in the *NF1* gene is not a frequent occurrence required for the development of dermal neurofibromas. In addition this study finds that dermal neurofibromas show genetic stability not only in the *NF1* gene, but also throughout the genome. Therefore, other factors that contribute to tumorigenesis in NF1 must be considered. The identification of these other factor(s)

responsible for the development of neurofibromas will provide therapeutic targets and possible markers for future malignant transformation.

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REFERENCES

Barker, D., Wright, E., Nguyen, K., Cannon, L., Fain, P., Goldgar, D., Bishop, D.T., Carey, J., Baty, B., Kivlin, J., et al (1987). Gene for Von Recklinghausen's neurofibromatosis in the pericentric region of chromosome 17. Science *236*, 1100-1102.

Basu, T.N., Gutmann, D.H., Fletcher, J.A., Glover, T.W., Collins, F.S., Downward, J. (1992) Abberant regulation of ras proteins in malignant tumour cells from type-1 neurofibromatosis patients. Nature *356*, 713-715.

Boguski, M.S. and McCormick, F. (1993). Proteins regulating Ras and its relatives. Nature 366, 643-654.

Bollag, G., Clapp, D.W., Shih, S., Adler, F., Zhang, Y.Y., Thompson, P., Lange, B.J., Freedman, M.H., McCormick, F., Jacks, T. and Shannon, K. (1996) Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells. Nat. Genet. 12, 144-148.

Cawthon, R., Weiss, R., Xu, G., Viskochil, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland, R., O'Connell, P., et al (1990) A major segment of neurofibromatosis type 1 gene: cDNA sequence, genomic structure and point mutations. Cell *62*, 193-201.

Coleman, S.D., Williams, C.A. and Wallace, M.R. (1995) Benign neurofibromas in type 1 neurofibromatosis (NF1) show somatic deletions of the NF1 gene. Nat. Genet. *11*, 90-92.

Däschner, K., Assum, G., Eisenbarth, I., Krone, W., Hoffmeyer, S., Wortmann, S., Heymer, B. and Kehrer-Sawatzki, H. (1997) Clonal origin of tumor cells in a plexiform neurofibroma with LOH in NF1 intron 38 and in dermal neurofibromas without LOH of the NF1 gene. Biochem. Biophys. Res. Comm. *234*, 346-350.

DeClue, J.E., Papageorge, A.G., Fletcher, J.A., Diehl, S.R., Ratner, N., Vass, W.C., Lowy, D.R. (1992). Abnormal regulation of mammalian p21^{ras} contributes to malignant tumor growth in von Recklinghausen (type-1) neurofibromatosis. Cell *69*, 265-273.

Eisenbarth, I., Beyer, K., Krone, W. and Assum G. (2000) Toward a survey of somatic mutation of the NF1 gene in benign neurofibromas of patients with neurofibromatosis type 1. Am. J. Hum. Genet. Feb;66(2):393-401.

Guha, A., Lau, N., Huvar, I., Gutmann, D., Provias, J., Pawson, T. and Boss, G. (1996) Ras-GTP levels are elevated in human NF1 peripheral nerve tumors. Oncogene *12*, 507-513.

John, A.M., Ruggieri, M., Ferner, R. and Upadhyaya, M. (2000) A search for evidence of somatic mutations in the NF1 gene. J. Med. Genet. 37, 44-49.

Legius, E., Marchuk, D., Collins, F.S. and Glover, T.W. (1993) Somatic deletion of the neurofibromatosis type 1 gene in a neurofibrosarcoma supports a tumour suppressor gene hypothesis. Nat. Genet. *3*, 122-126.

Li, Y., O'Connell, P., Breidenbach, H.H., Cawthon, R., Stevens, J., Xu, G., Neil, S.M., Robertson, M., White, R., Viskochil, D. (1995). Genomic organization of the neurofibromatosis 1 gene (NF1). Genomics 25, 9-18.

Martin, G.A., Viskochil, D., Bollag, G., McCabe, P.C., Crosier, W.J., Haubruck, H., Conroy, L., Clark, R., O'Connell, P.O., Cawthon, R.M., Innis, M.A. and McCormick, F. (1990) The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. Cell 63: 843-849.

Menon, A.G., Anderson, K.M., Riccardi, V.M., Chung, R.Y., Whaley, J.M., Yandell, D.W., Farmer, G.E., Freiman, R.N., Lee, J.K., Li, F.P., Barker, D.F., Ledbetter, D.H., Kleider, A., Martuza, R.L., Gusella, J.F. and Seizinger, B.R. (1990) Chromosome 17p deletions and p53 gene mutations associated with the formation of malignant neurofibrosarcomas in von Recklinghausen neurofibromatosis. Proc. Natl. Acad. Sci. U.S.A. 87, 5435-5439.

Paulson, T.G., Galipeau, P.C. and Reid, B.J. (1999) Loss of heterozygosity analysis using whole genome amplification, cell sorting, and fluorescence-based PCR. Genome Res. 9: 482-491.

Purandare, S.M., Breidenbach, H.H., Li, Y., Zhu, X.L., Sawada, S., Neil, S., Brothman, A., White, R., Cawthon, R. and Viskochil, D. (1995) Identification of neurofibromatosis 1 (NF1) homologous loci by direct sequencing, fluorescence in situ hybridization, and PCR amplification of somatic cell hybrids. Genomics 30, 476-485.

Purandare, S.M., Cawthon, R., Nelson, L.M., Sawada, S., Watkins, W.S., Ward, K., Jorde, L.B., and Viskochil, D.H. (1996) Genotyping of PCR-based polymorphisms and linkange-disequilibrium analysis at the NF1 locus. Am. J. Hum. Genet. 59: 159-166.

Rasmussen, S.A., Overman, J., Thomson, S.A., Colman, S.D., Abernathy, C.R., Trimpert, R.E., Moose, R., Virdi, G., Roux, K., Bauer, M., Rojiani, A.M., Maria, B.L., Muir, D., Wallace, M.R. (2000) Chromosome 17 loss-of-heterozygosity studies in benign and malignant tumors in neurofibromatosis type 1. Genes Chromosomes Cancer 28: 425-431.

Riccardi, VM. (1992). Neurofibromatosis: phenotype, natural history, and pathogenesis. Johns Hopkins University Press, Baltimore.

Seizenger, B.R., Rouleau, G.A., Ozelius, Z., Lane, A.H., Faryniarz, A.G., Chao, M.V., Huson, S., Korf, B.R., Parry, D.M., Pericak-Vance, M.A., et al (1987). Genetic linkage of von Recklinghausen's neurofibromatosis to the nerve growth factor receptor gene. Cell 49, 589-594.

Serra, E., Puig, S., Otero, D., Gaona, A., Kruyer, H., Ars, E., Estivill, X. and Lázaro, C. (1997) Confirmation of a double-hit model for the NF1 gene in benign neurofibromas. Am. J. Hum. Genet. 61, 512-519.

Shannon, K.M., O'Connell, P., Martin, G.A., Paderanga, D., Olson, K., Dinndorf, P. and McCormick, F. (1994) Loss of the normal NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders. N. Engl. J. Med. 330, 597-601.

The Utah Marker Development Group. (1995) A collection of ordered tetranucleotide-repeat markers from the human genome. Am. J. Hum. Genet. 57: 619-628.

Viskochil, D., Buchberg, A.M., Xu, G., Cawthon, R.M., Stevens, J., Wolff, R.K., Culver, M., Carey, J.C., Copeland, N.G., Jenkins, N.A., et al. (1990). Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. Cell 62, 187-192.

Wallace, M.R., Marchuk, D.A., Anderson, L.B., Letcher, R., Odeh, H.M., Saulino, A.M., Fountain, J.W., Brereton, A., Nicholson, J., Mitchell, A.L., et al. (1990). Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three patients. Science 249, 182-186.

Xu, W., Mulligan, L.M., Ponder, M.A., Liu, L., Smith, B.A., Mathew, C.G. and Ponder, B.A. (1992) Loss of NF1 alleles in phaeochromocytomas from patients with type I neurofibromatosis. Genes Chrom. Cancer 4, 337-342.

Figure 1. Map of location of dermal neurofibromas found on the body of B100. Asterisks denote positions of single neurofibromas and circles denote groups of neurofibromas.

Table 1. List and sequence of primers used to amplify the 60 exons of the *NF1* gene.

Figure 2. MS-PCR of positions 702, 2034 and 10647 in the *NF1* gene. The negative control is denoted by the dH20 lane, and the positive controls are indicated by A, G or AG at each position. DNA from each patient is indicated under the respective lane.

Figure 3. Size shift mutation analysis of the *NF1* gene. The patient samples are indicated across the top of the gel. In this analysis products from exon 18 and 19a were multiplexed. In this particular autoradiograph the DNA from U125 blood did not amplify.

Figure 4. Haematoxylin and eosin stain of neurofibroma D from patient U135. a) Shows the entire neurofibroma section. b) Shows the area of skin that was microdissected. c) Shows area DA that was microdissected. d) Shows area DB that was microdissected.

Figure 5. Densitometry of neurofibromas from patient U135. a) Allelic imbalance analysis of DNA extracted from neurofibroma tissue. The dashed lines indicate the cutoff values of 75% and 125% respectively. b) Allelic imbalance analysis of DNA extracted by LCM from sections of dermal neurofibromas. The dashed lines indicate the cutoff values of 75% and 125% respectively. Results are from 4 experiments. Inset into each graph are a corresponding autoradiograph.

Table 2. Genotypes of 19 dermal neurofibromas from 4 out of the 5 patients. The markers used are indicated along the side of the table, while patient samples are across the top of the table. Symbols used are as follows. = allelic balance; F failed sample; I allelic imbalance; N not informative.
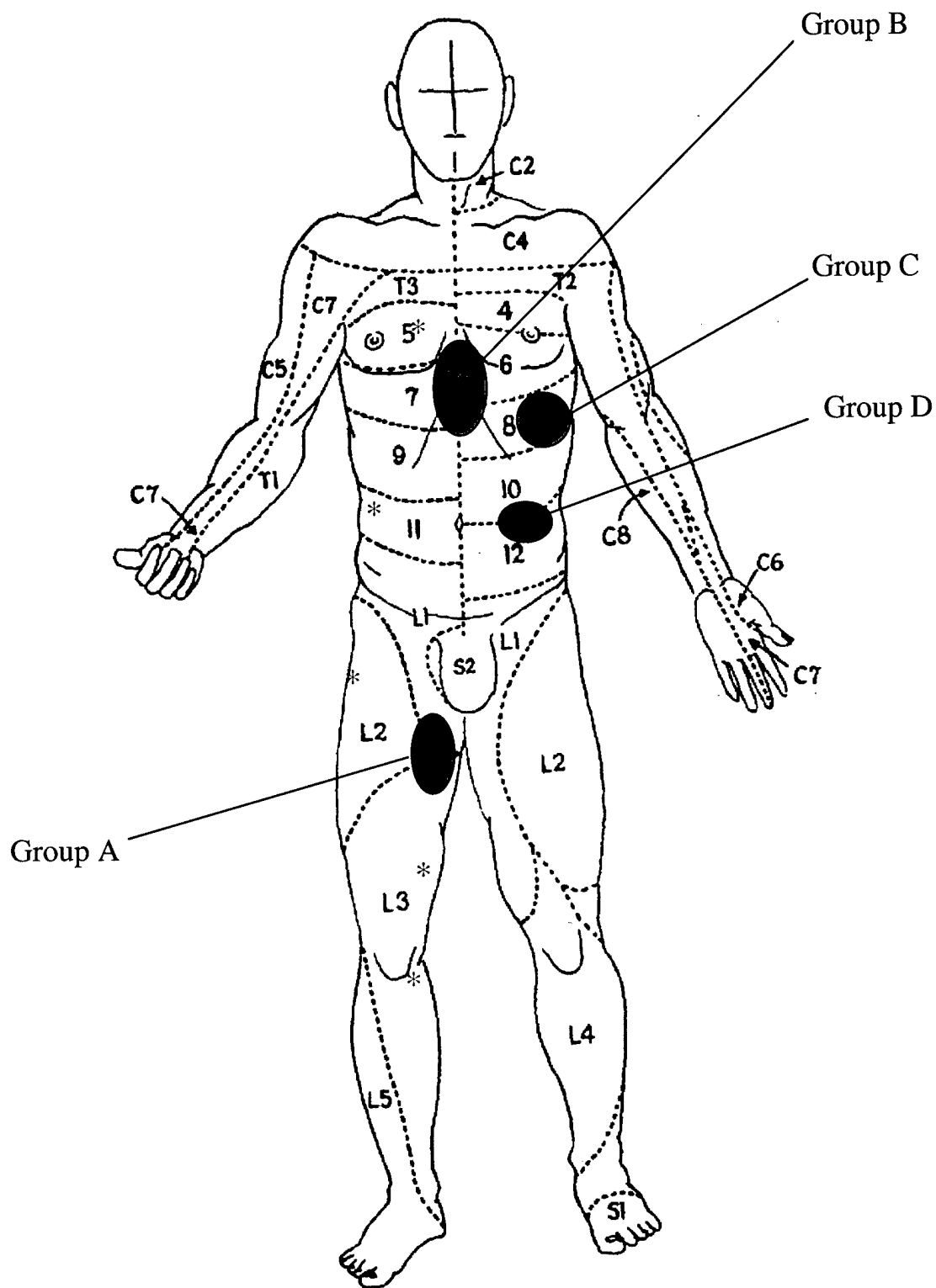
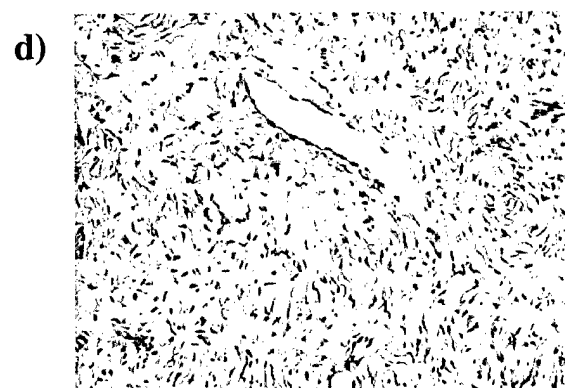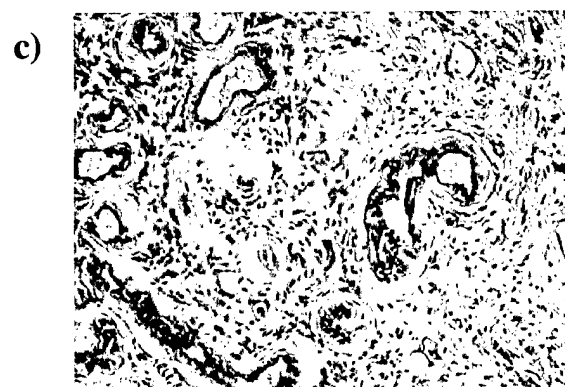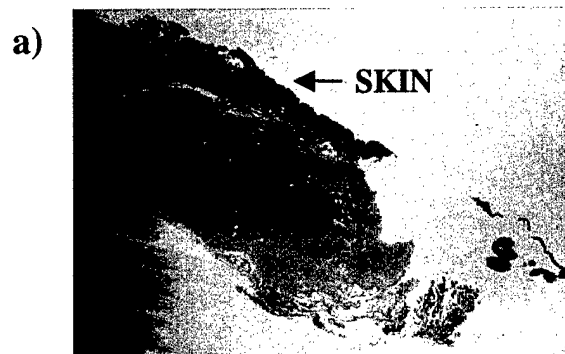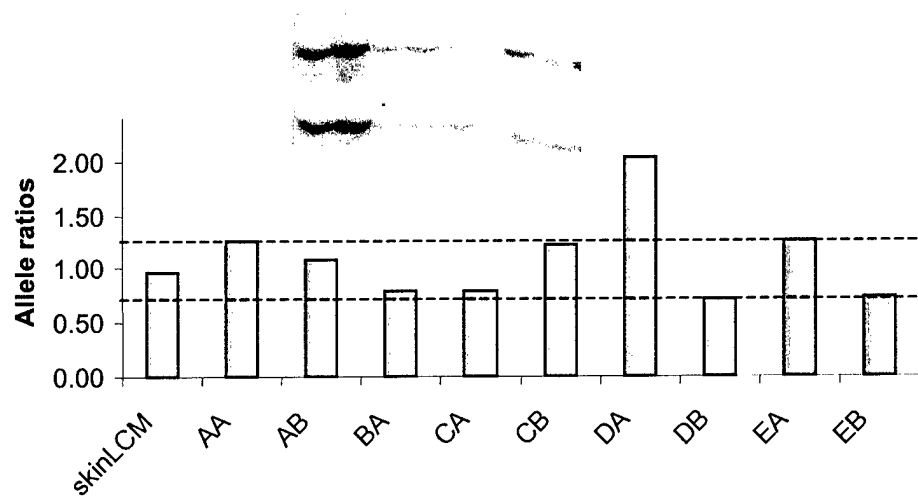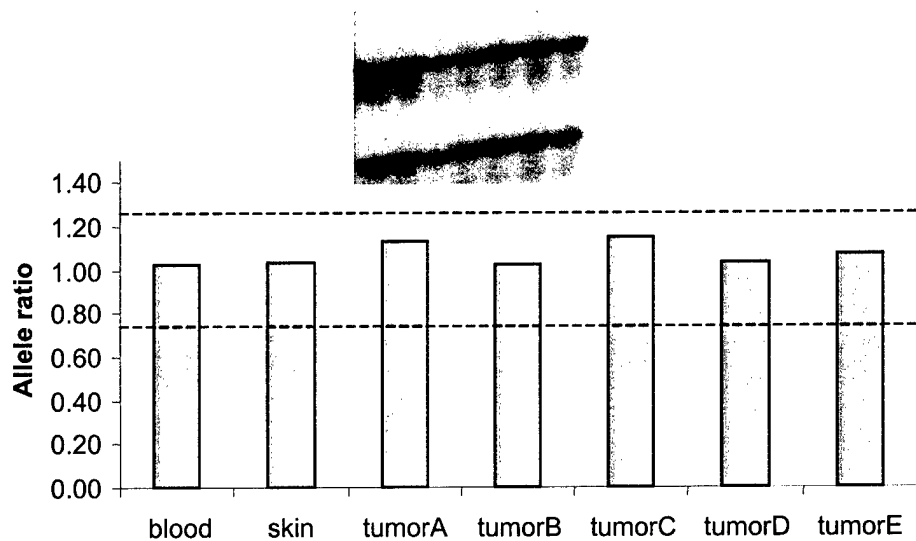| Exon(bp)	Sequence (5'→3')	Exon(bp)	Sequence (5'→3')
1 (438)	CAGACCCTCTCCTTGCCTCTT GGATGGAGGGTCGGAGGCTG	21 (374)	AAATGAAAGTTTCATATAGAAATAC ATTTGCTATGTGCCAGGGAC
2 (221)	TTTTAAGGATAAACTGTTTACGTG TCCCCAAAACACAGTAACCC	22 (331)	TGCTACTCTTTAGCTTCCTAC CCTTAAAAGAAGACAATCAGCC
3 (241)	TTTCACTTTTCAGATGTGTGTTG TGGTCCACATCTGTACTTTGG	23-1 (281)	TTTGTATCATTCATTTGTGTGTA AAAAACACGGTTCTATGTGAAAAG
4a (415)	TTTGAAAATTTTCATAATAGAAATGT GAGGTCAAAGCTGCTGTGAG	23-2 (268)	CTTAATGTCTGTATAAGAGTCTC ACTTTAGATTAATAATGGTAATCTC
4b (339)	CTCCTGGCTCAAGTGGTC TTATAAAATCCAGATTGGTGTTC	23a (412)	CCAGAAATAGTATACATGATTGGG ACAGTGCAGGTCAAATAGGCTG
4c (283)	TTTCCTAGCAGACAACTATCGA CATCAAAAAAAAAATTTTAATACCAG	24 (266)	TTGAACTCTTTGTTTTCATGTCTT GGAATTTAAGATAGCTAGATTATC
5 (240)	TGCTTGAGTGATAGTTTCACAT AAAAAAAAATCAATCGTATCCTA	25 (338)	AATATAATAATTATATTTGGGAAGGT GAAAATATTTGATTCAAACAGAGC
6 (301)	CATGTTATCTTTTAAAAATGTTGCC ATAATGGAAATAATTTTGCCCTCC	26 (342)	GCTTTGTCTAATGTCAAGTCAC TTAAACGGAGAGTGTTCACTATC
7 (373)	TGCTATAATATTAGCTACATCTGG CCTATGAACTTATCAACGAAGAG	27a (298)	GTTACAAGTTAAAGAAATGTGTAG CTAACAAGTGGCCTGGTGGCAAAC
8 (318)	TGTGCTGCTTCTGGCAACTG CTAGTCTTTCTGTTTATAAAGGAT	27b (296)	TTTATTGTTTATCCAATTATAGACTT TCCTGTTAAGTCAACTGGGAAAAAC
9 (248)	TTTGACCTCATTTGTATTACTGAG AGAACCTTTTGAAACCAAGAGTG	28.1 (334)	CATCTGTATATTTATTTTAAACACTG CTACCTTTGAGGCCAGTCAG
9a (294)	TCCGCTGTGGCTCAGAACAC AGTAGAAGAGGATGCACAGCC	28.2 (271)	GAGTACACCAAGTATCATGAG AGGATATAGTCTAGTTAGTCAAG
10a (232)	ACGTAATTTTGTACTTTTTCTTCC CAATAGAAAGGAGGTGAGATTC	29 (530)	CCTTAAATGGCATAGTGTTTTGT TTTTAAAATTCATTAAATGGTCTCA
10b (267)	CTTTAAAGTGATAGCTATTACC TCTTGGCGATTCAGCTAAACC	30 (367)	GATCATACTTTGTAACAGAATCACA GGGTTTTCTTTGAATTCTCTTAGA
10c (379)	CTTGGTACCCTTTAGCAGTCAC CCTTCTTTCTCCATGGAG	31 (324)	CCGAATTCTTTATGTTAAATAATTG AGATAAATATGTGCACAAAGGAG
11 (189)	GTACTCCAGTGTTATGTTTACC TAAAGTTGAAATTTAAAAATTAAAGTAC	32 (312)	ATCTAGTATTTTTGAGGCCTCAG CAGATATGCTATAGTACAGAAGG
12a (303)	AAACCTTACAAGAAAAACTAAGCT ATTACCATTCCAAATATTCTTCCA	33 (462)	CATATCTGTTTTATCATCAGGAGG AAGTAAAATGGAGAAAGGAACTGG
12b (382)	TTTCTAGTGAATCTCCTTCAAGT ATGAAATTTACCAAATTTCATTCAG	34 (400)	CAAAATGAAACATGGAACTTTAGA TAAGCATTAAGTACAAAATAGCACA
13 (382)	CACAGTTTATTGCATTGTTAGAT GCCATGTGCTTTGAGGCAGAA	35 (311)	ATAGAAGAAATATTGGTTTATTGTG ACATTGATCATACAGTAATAGCTG
14 (285)	TCCTTTTGGGTGGAGCTTATC TATACTTGTAATATGCACGTATC	36 (278)	AAATACAGCTATTACTGTATGATCA AATGAAAGATATGCTTTACACTTGA
15 (275)	TGTGATCAGGAATAGCTTTTGAA TTAACAGATAAAAGTCAACTTTAC	37 (318)	AGAAAACAAATGTACATTAAGCTAG CAACACTGATACCCAAAATGAATG
16 (549)	TGGATAAAGCATAATTTGTCAAGT TAGAGAAAGGTGAAAAATAAGAG	38 (362)	ACATATGGAAAAGTGAAGAGCTTA AAGATGGAAGAGTACTAAATTTGA
17 (318)	CTCTGTGTGTTTAGATCAGTCA TTTATCAATTACTACCAGTAGCAG	39 (382)	CTTGCACCAGTTAATTTGTAGTAG AATCACTTATTCAAATTACTTCTGG
18 (367)	AGAAGTTGTGTACGTTCTTTTCT CTCCTTTCTACCAATAAGCGC	40 (328)	TCAGGGAAGAAGACCTCAGCAGATGC TGAACTTTCTGCTCTGCCACGCAACC
19a (267)	TCATGTCACTTAGGTTATCTGG TGTAATTAAGTAGTTATAACTCTC	41 (339)	TTATGTAGTCTTCCAAAATATGTG TTGCCTCCATTAGTTGGAAAATTG
19b (276)	CCTAAAGTTTATATCTGTTAATAAG TGGTGGGGGGCTTTATTTGC	42 (356)	CTTGGAAGGAGCAAACGATGGTTG CAAAAACTTTGCTACACTGACATGG
20 (402)	CCACCCTGGCTGTTATCG TAATTTTTGCTTCTCTTACATGC	43 (318)	GCTCCAGGGATGTATTAGAGCTTTC CATGTACTCTCCCACCTTATTTTC
44 (334)	ATACAGCATTGTAAATAGGTAGCC AAAATTTGAGGGTGGGGGACTC	48a (245)	ATCTAGTATCTAATTGTATTTCACC GCAGACTGAGCTTACAGGGAC
45 (341)	TCCCCTTTTTGAGTCCCCCACC CACATTACTGGGTAAGCATTTAAC	49.1 (468)	CAGAACAACTGCAAAGAAAGTG CCCACTTTCTTTGCAGTTGTTCTG
46 (347)	ATTTGGAAAATGAAGAAATGCCCC ATGTTAGCAAGTTCATCAACCATC	49.2 (326)	TTCTGGGGTAAGTTTCACAGTTTC TTTCAAACTCCTAGGATGTACTTAC
47 (313)	TCTCAACTGTATGTCCAATGTAAC TGTGTGTTCTTAAAGCAGGCATAC	49.3 (435)	GTAAAGCAGTTAGTTGCTGCAC GGGAAGAAAACAGAGCCGAGAC
48 (360)	TAATATTTTTGGCTTCAGATGGGG CACAAGGGAATTCCTAATGTTGGTG		

Position 702   Position 2034   Position 10647

dH₂O | AG | A | G | B100 | U100 | U125 | U129 | U135 | dH₂O | G | A | AG | B100 | U100 | U125 | U129 | U135 | dH₂O | G | AG | A | B100 | U100 | U125 | U129 | U135

a) ← SKIN

b) SKIN

c) DA

d) DB
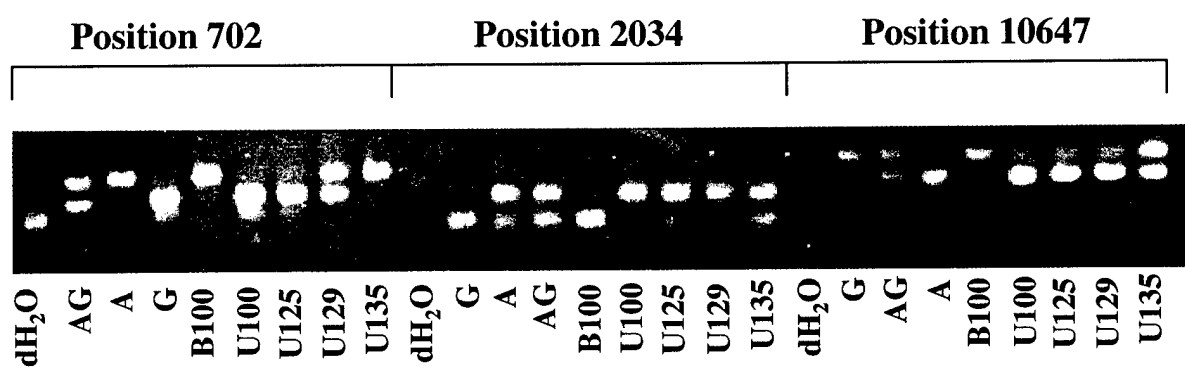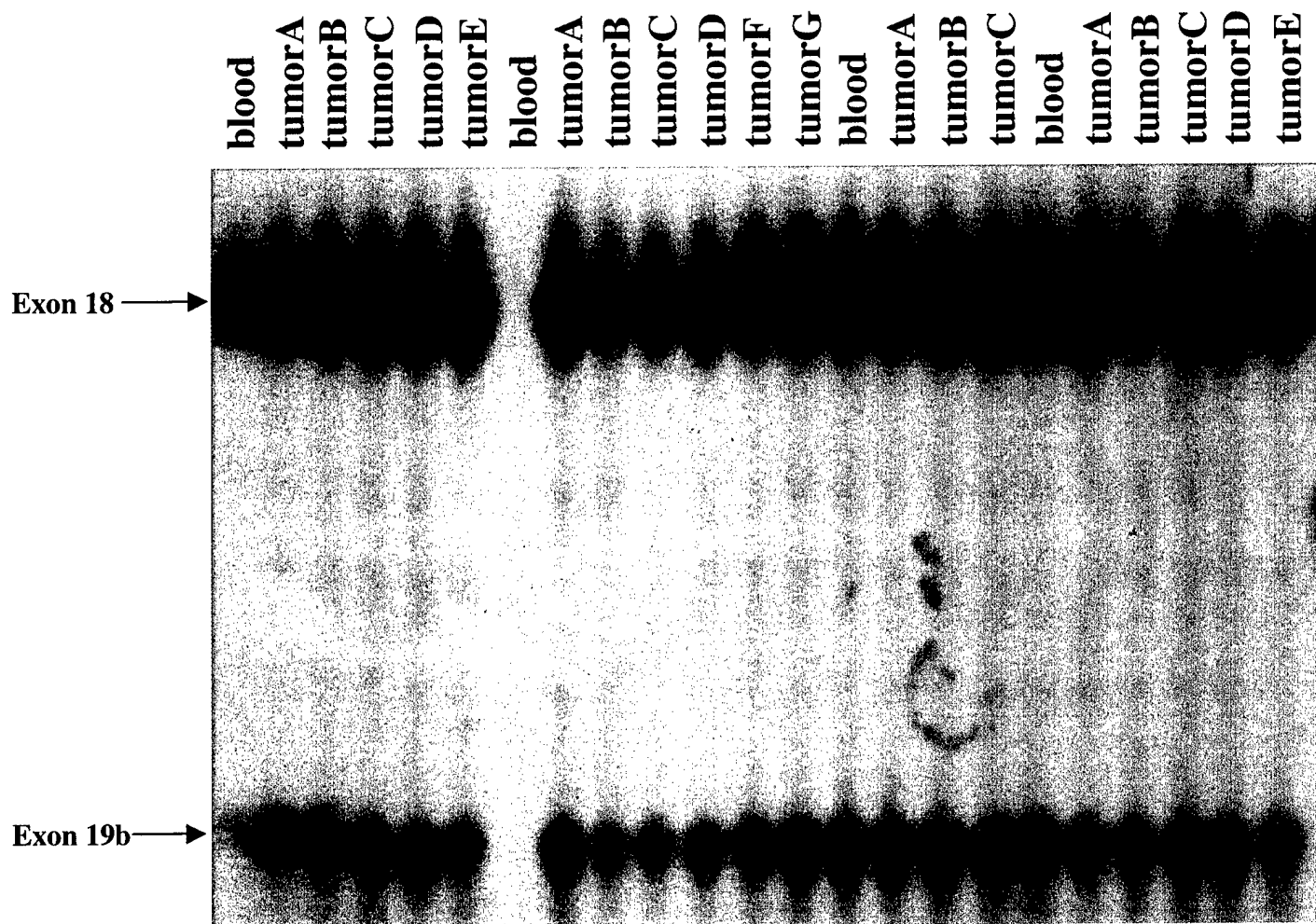
Marker	U100						U125							U129				U135					
	Blood	Tumor A	Tumor B	Tumor C	Tumor D	Tumor E	Blood	Tumor A	Tumor B	Tumor C	Tumor D	Tumor F	Tumor G	Blood	Tumor A	Tumor B	Tumor C	Blood	Tumor A	Tumor B	Tumor C	Tumor D	Tumor E
UT5144-1p		=	=	=	=	=		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT5170-1q		=	=	=	F	=		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT595-2p		F	F	F	F	=		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT1360-3p		=	=	=	=	=		=	=	=	=	=	=		=	F	=		=	=	=	=	=
UT6129-3q		=	=	=	=	=		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT878-4p		=	-	=	=	=		N	N	N	N	N	N		=	=	=		=	F	=	=	=
UT615-5p		=	=	=	=	=		=	=	=	=	=	=		N	N	N		=	=	=	=	=
UT5013-5q		=	=	=	=	=		=	=	=	=	=	=		=	=	=		F	=	F	=	=
UT886-5q		=	=	=	=	=		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT2018-6p		=	=	=	=	=		N	N	N	N	N	N		=	=	=		=	=	=	=	=
UT897-6q		N	N	N	N	N		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT5189-7p		=	=	=	=	=		=	-	=	=	=	=		=	=	=		=	F	=	=	=
UT5412-8p		=	=	=	=	=		=	=	=	=	=	=		=	=	=		N	N	N	N	N
UT909-8q		N	N	N	N	N		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT873-9p		=	=	=	=	=		=	=	=	=	=	=		=	=	=		F	=	F	=	=
UT913-9q		=	I	=	I	=		=	-	=	=	=	=		=	I	=		F	=	F	F	F
UT7422-9q		N	N	N	N	N		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT1699-10p		=	=	=	=	=		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT5419-10q		=	=	=	=	=		=	=	=	=	=	=		=	=	=		F	=	F	=	=
UT8115-11p		=	=	=	=	=		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT5150-11		=	=	=	=	=		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT2095-11q		=	=	=	=	=		N	N	N	N	N	N		N	N	N		N	N	N	N	N
UT5029-12p		N	N	N	N	N		=	=	=	=	=	=		N	N	N		=	=	=	=	=
UT6136-12		N	N	N	N	N		=	=	=	=	=	=		=	=	F		=	=	F	=	=
UT931-12q		N	N	N	N	N		=	=	=	=	=	=		=	=	=		N	N	N	N	N
UT2413-13		=	=	=	=	=		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT1392-14		=	=	=	=	=		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT1232-15		=	=	=	=	=		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT581-16p		=	=	=	=	=		=	=	=	=	=	=		-	=	=		N	N	N	N	N
UT703-16q		=	=	=	=	=		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT269-17p		=	=	=	=	=		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT40-17q		N	N	N	N	N		=	=	=	=	=	F		N	N	N		=	=	F	=	I
UT7162-18p		=	=	F	=	F		=	=	=	=	=	=		=	=	=		=	=	=	=	=
UT576-18q		=	=	=	=	=		N	N	N	N	N	N		=	=	=		=	=	F	=	=
UT5187-19p		=	=	=	=	=		=	=	=	=	=	=		=	=	=		N	N	N	N	N
UT1342-19q		=	=	=	=	=		=	=	=	=	=	=		N	N	N		=	=	=	=	=
UT236-20		F	F	F	F	F		F	F	F	F	F	F		=	=	=		=	=	=	=	=
UT1355-20q		=	=	=	=	=		=	=	=	=	=	=		F	F	F		=	=	=	=	=
UT1091-22q		=	=	=	=	=		=	=	=	=	=	=		=	=	=		=	=	=	=	=